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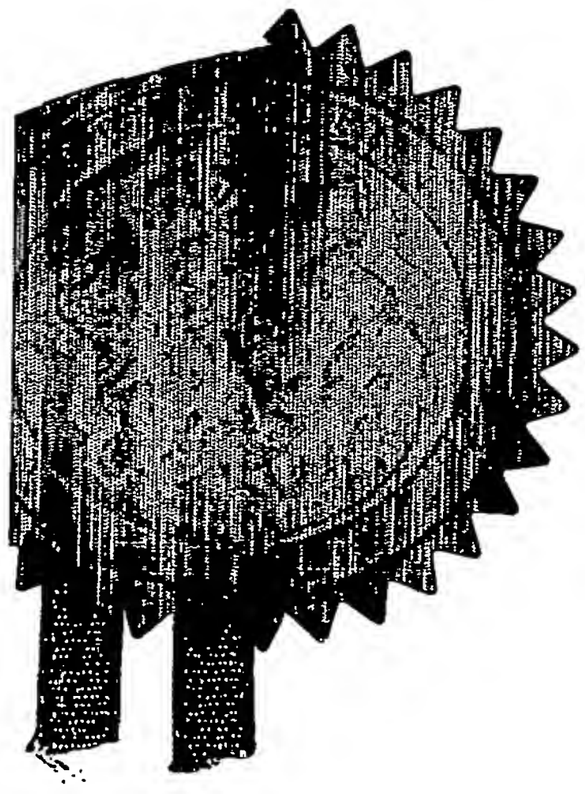

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3. Full name, address and postcode of the or of each applicant *(underline all surnames)* — **Clasado Inc.**
Edificio Banco do Brazil
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Panama 5
Panama

Patents ADP number *(if you know it)* — 08664203001

If the applicant is a corporate body, give the country/state of its incorporation — **Panama**

4. Title of the invention — **Novel Galactooligosaccharide Composition and the Preparation Thereof**

5. Name of your agent *(if you have one)* — **Saunders & Dolleymore**

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)* — **9 Rickmansworth Road**
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NOVEL GALACTOOLIGOSACCHARIDE COMPOSITION AND THE PREPARATION THEREOF.

The present invention relates to novel strains of *Bifidobacterium bifidum* that produce a novel β-galactosidase enzyme activity capable of converting lactose to a novel mixture of galactooligosaccharides. Galactooligosaccharides are non-digestible carbohydrates, which are resistant to mammalian gastrointestinal digestive enzymes but are fermented by specific colonic bacteria. The invention also relates to the use of a bifidobacterial strain to produce a novel galactooligosaccharide composition that is capable of promoting the growth of bifidobacteria in the gut. It also relates to the novel composition of the galactooligosaccharide products.

The human gut flora comprises pathogenic, benign and beneficial microbial genera. A predominance of the former can lead to intestinal disorders that can be both acute (e.g. gastroenteritis) and chronic (e.g. inflammatory bowel disease and some intestinal cancers). Attempts have been made to influence the balance of the gut flora in favour of beneficial microorganisms, such as the bifidobacteria, by adding one or more such microbial strains to an appropriate food vehicle. Such a live microbial feed supplement is known as a probiotic. However, it is difficult to guarantee the survival of live bacteria in foods and also after digestion.

An alternative approach to dietary manipulation of the gut microflora is the use of a prebiotic, which is defined as a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thereby resulting in an improvement in the health of the host.

The human large intestinal microflora is acquired at birth. The breast-fed infant has a preponderance of bifidobacteria, which easily out compete other genera. This is because human milk components are stimulatory. In contrast, the formula-fed infant has a more

complex flora which resembles the adult gut in that bacteroides, clostridia, bifidobacteria, lactobacilli, gram positive cocci, coliforms and other groups are all represented in fairly equal proportions. Bifidobacteria are generally regarded as protective with regard to the large intestinal infections and this difference probably explains the much lower incidence of infection in breast fed infants compared to those who are fed on formula milk.

Certain components of the gut flora have been implicated in the aetiology of gut disease. For example, mycobacteria are associated with Crohn's disease, ulcerative colitis may be triggered by sulphate reducing bacteria and there may be bacterial involvement in the development of bowel cancer. It would clearly be of benefit if the selective growth of indigenous beneficial gut bacteria could be encouraged by the ingestion of a prebiotic. This would have the ongoing effect that the pathogenic microflora would be repressed.

One group of compounds that are classified as prebiotics is the galactooligosaccharides which are galactose-containing oligosaccharides of the form Glc α1-4[β Gal 1-6]$_n$ where n=2-5, and are produced from lactose syrup using the transgalactosylase activity of the enzyme β –galactosidase (Crittenden, (1999) Probiotics: A Critical Review. Tannock, G.(ed) Horizon Scientific Press, Wymondham, pp. 141-156). Three products are currently commercially available having slightly different compositions. The first of these, transgalactosylated oligosaccharides (TOS), is produced using β-galactosidase from *Aspergillus oryzae* (Tanaka *et al*, (1983) Bifidobacteria Microflora, 2, 17-24), and consists of tri-, tetra-, penta- and hexa-galacto-oligosaccharides. The second is Oligomate 55, which is prepared using β-galactosidase from *A. oryzae* and *Streptococcus thermophilus* (Ito *et al.*, (1990), Microbial Ecology in Health and Disease, 3, 285-292) and contains 36% tri-, tetra-, penta- and hexa-galacto-oligosaccharides, 16% disaccharides galactosyl glucose and galactosyl galactose, 38% monosaccharides and 10% lactose. Finally,a transgalactosylated disaccharide (TD) preparation is produced using β-galactosidase from *S. thermophilus* (Ito *et al.*, (1993),J. Nutritional Science and Vitaminology, 39, 279-288).

It is known that members of the bifidobacteria produce β-galactosidase enzymes that are involved in the bacterial metabolism of lactose. Moller, P.L. *et al* in Appl. & Environ. Microbiol., (2001),62,(5), 2276-2283 describe the isolation and characterization of three β-galactosidase genes from a strain of *Bifidobacterium bifidum.*

US patent publication No. US 2002/0086358 describes a new β-galactosidase from *Bifidobacterium bifidum*, in particular a truncated version of the enzyme that has a high transgalactosylating activity. Whilst it was stated that incubation with lactose could take place in the presence of 0.5-60% lactose, the maximum exemplified yield of galactooligosaccharide produced in transgalactosylation reactions was 44% (mg of oligosaccharide produced per mg lactose added). Moreover, from the definition of oligosaccharide in this US patent publication it is evident that the product consists of at least three linked sugar molecules.

Strains of Bifidobacterium have now been found that are not only capable of producing a β-galactosidase enzyme activity that converts lactose to a mixture of galactooligosaccharides, but also produce a galactooligosaccharide mixture which contains up to 35% of the disaccharide galobiose. The latter is known (see Paton, J.C. & Paton, A.W. (1989), Clin. Microbiol. Revs., 11, 450-479; Carlsson, K.A. (1989), Ann. Reviews Biochem., 58, 309-350.) to be an antiadhesive capable of preventing the adhesion of toxins, e.g. Shiga toxin, and pathogens, such as *E. coli*, to the wall of the gut.

According to the invention there is provided a strain of *Bifidobacterium bifidum* capable of producing a β-galactosidase enzyme activity that converts lactose to a mixture of galactooligosaccharides comprising a disaccharide, at least one trisaccharide, a tetrasaccharide and a pentasaccharide. Preferably the mixture comprises from 20 to 35% w/v of the disaccharide, from 20 to 35% w/v of the trisaccharide(s), from 15 to 25% w/v of the tetrasaccharide and from 10 to 20% w/v of the pentasaccharide.

The term enzyme activity", as used in relation to the β-galactosidase enzyme

activity of the present invention, is the activity resulting from at least one β-galactosidase enzyme.

In one aspect, the galactooligosaccharide mixture has been found to comprise the disaccharide Gal-Gal, the trisaccharides Gal-Gal-Glc and Gal-Gal-Gal, the tetrasaccharide Gal-Gal-Gal-Glc and the pentasaccharide Gal-Gal-Gal-Gal-Glc, where Gal represents a galactose residue and Glc represents a glucose residue.

Using methylation analysis the galactooligosaccharide mixture has been found to comprise Gal (β1-6)-Gal (β1-6)-Gal (β 1-4)-Glc tetrasaccharide; Gal (β1-6)-Gal (β1-4)-Gal and Gal (β1-3)-Gal (β1-4)-Glc trisaccharides; Gal (β1-3)-Glc and Gal (β1-3)-Gal and Glc (β1-6)-Gal and Gal (α 1-6)-Gal disaccharides.

A strain of *Bifidobacterium bifidum* capable of producing a β-galactosidase enzyme activity that converts lactose to the mixture of galactooligosaccharides as defined above has been deposited under accession number NCIMB 41171 at the National Collection of Industrial and Marine Bacteria, Aberdeen on 31 March 2003.

Such a deposited strain of *Bifidobacterium bifidum,* or its biologically functional equivalent, can be used to produce the galactooligosaccharide mixture as defined above. The mixture of galactooligosaccharides may form part of a product for improving gut health by promoting the growth of bifidobacteria in the gut, specifically the origin producer strain. Such a product may be selected from the group consisting of dairy products (for example, liquid milk, dried milk powder such as whole milk powder, skimmed milk powder, fat filled milk powders, whey powders, baby milks, ice cream, yoghurt, cheese, fermented dairy products), beverages, infant foods, cereals, bread, biscuits, confectionary, cakes, food supplements, dietary supplements, animal feeds, poultry feeds or indeed any other food or beverage.

The phrase "biologically functional equivalent" is construed to mean a strain of

Bifidbacterium bifidum that is capable of producing a β-galactosidase enzyme activity that converts lactose into the mixture of galactooligosaccharides as defined above.

According to another aspect of the invention there is provided a galactooligosaccharide composition for promoting growth of bifidobacteria comprising as effective constituents a disaccharide, at least one trisaccharide, a tetrasaccharide and a pentasaccharide.

Preferably the galactooligosacharide composition comprises from 20 to 35% w/v of the disaccharide, from 20 to 35% w/v of the trisaccharide(s), from 15 to 25% w/v of the tetrasaccharide and from 10 to 20% w/v of the pentasaccharide.

According to yet another aspect of the invention there is provided a method for the manufacture of a substance for promoting the growth of bifidobacteria characterised in that lactose or a lactose-containing material is treated with a strain of *Bifidobacterium bifidum* as defined above.

Suitable lactose-containing material may be selected from commercially available lactose, whole milk, semi-skimmed milk, skimmed milk, whey and fat-filled milk. Such milk products may be obtained from cows, buffalos, sheep or goats. Fat-filled milk is defined as whole milk that has been skimmed to remove the dairy fat, which is subsequently replaced by the addition of vegetable fat or oil.

Using growth media supplemented with carbohydrate substrates other than lactose it has been found that *Bifidobacterium bifidum* according to the invention can utilise maltose, raffinose, xylan and fructose. Culturing of the bacteria in medium supplemented with one of these carbohydrates induced the expression of α-glucosidase, α-galactosidase, xylosidase and β-fructofuranosidase respectively and thus resulted in the production of α-glucooligosaccharides, α-galactooligosaccharides, xylooligosaccharides and fructooligosaccharides respectively.

In an investigation leading to the present invention, gut derived bacteria were screened for those that were capable of producing β-galactosidase and thus had the highest potential for producing galactooligosaccharide(s). As a result, it has been found that certain bacteria belonging to the genus Bifidobacterium, in particular *Bidobacterium bifidum,* were able to not only produce a β-galactosidase enzyme activity but also that the enzyme could convert lactose to a galactooligosaccharide mixture comprising from 20 to 35% w/v of a disaccharide, from 20 to 35% w/v of trisaccharide, from 15 to 25% w/v of a tetrasaccharide, from 10 to 20% w/v of a pentasaccharide. A specific example of *Bifidobacterium bifidum* was deposited on 31 March 2003 with NCIMB, Aberdeen under accession number 41171.

In order to culture these bacteria, any nutrient source can be utilized provided it can be assimilated by the bacteria. Appropriate culture media can be formulated with, for example, carbohydrates such as lactose, sucrose or glucose; nitrogen containing inorganic or organic nutrient sources such as yeast extract, tryptone, meat extract (Lab Lemco) and the like; inorganic nutrient sources such as phosphates, potassium and the like. For culturing, the pH of the nutrient medium should be within the range of 6.0 to 8.0. preferably 7.0 and culturing is carried out anaerobically at a temperature range of from 35° to 40° C, preferably 37° C for from 40 to 64 hours, preferably 50 hours.

The strain can be cultured by any of the known cultural methods such as stationary phase culture, anaerobic submerged culture or shake culture. The bacterial cells are harvested by centrifugation or filtration and, following resuspension in 100% ethanol and washing in a suitable buffer, the cells can be used as such as the reaction catalyst without further treatment. As an alternative the cells may be used in an immobilized state by an appropriate immobilization procedure.

Once immobilized, the *Bifidobacterium bifidum* of the invention may be used to convert lactose itself or lactose contained in a milk product into the novel galactooligosaccharide composition of the invention.

Milk containing the galactooligosaccharide composition of the invention produced in this way may be administered directly to children, adults or animals. Alternatively, it may be used to produce products such as bread, confectionary or the like, where the stability of galactooligosaccharides under acidic and high temperature conditions enables it to be used without decomposition.

Alternatively, the galactooligosaccharide composition of the invention may be mixed with a culture of the *Bifidobacterium bifidum* according to the invention to produce a mixture for improving gut health. Such a mixture is classed as a synbiotic, which is defined as 'a mixture of probiotic and prebiotic that beneficially affects the host by improving the survival and implantation of live microbial dietary supplement in the GI tract' (see Gibson and Roberfroid, 1995, Dietary modulation of the human microbiota: introducing the concept of prebiotics. Journal of Nutrition 125, 1401-1412). Such a combination enhances the survival of the probiotic in the hostile environment of the colon by offering an available selective substrate. The bacterial probiotic may be microencapsulated in the galactooligosaccharide prebiotic to produce, for example, a powder, which may then be added to dairy products, such as yoghurt, or used as a dietary supplement.

The advantage of ingesting milk or other products containing the galactooligosaccharide composition of the invention is it promotes an increase in the levels of beneficial bifidobacteria in the gut, at the expense of other less desirable bacteria present in the gut micoflora, such as the clostridia. Thus, there is a decrease in certain indigenous bacteria that could have a deleterious effect upon the health of the individual. This would then result in a reduction of gastrointestinal tract infections. It helps to prevent colitis, shortens diarrhoeal incidents and reduces the risk of chronic gut diseases such as ulcerative colitis and cancer.

The present invention will be further described by way of reference to the following examples.

EXAMPLE 1

1l of medium (pH 7.0) containing 10.0g/l tryptone. 5.0 g/l Lab-LEMCO (meat extract), 5.0 g/l yeast extract, 3.0 g/l K HPO , 0.05 g/l cysteine HCL, 10 g/l lactose and 1ml/l Tween 80 was sterilized at 121°C for 15 min. After sterilization the medium was inoculated with 1.0% (v/v) of a fresh *Bifidobacterium bifidum* NCIMB 41171 culture and incubated under anaerobic condition at 37°C for 50h. The bacterial cells were harvested by centrifugation (30000 g for 20 min) and resuspended in 100% ethanol for 15 min. After being washed twice with phosphate buffer (0.02M. pH 7.0) the fixed cells were ready to be used in oligosaccharide synthesis reactions.

The bacterial cells (40 units of β-galactosidase activity) were resuspended in 100ml of phosphate buffer (0.02M. pH 7.0) containing 50 g of lactose. The reaction was allowed to proceed at 40°C and after 7h the mixture consisted of 35% (w/v) hydrolysis products (glucose, galactose), 37% (w/v) lactose and 18% (w/v) galactooligosaccharides with a degree of polymerisation between 2-6. products were quantitatively analysed by high performance liquid chromatography using a Merck-Hitachi LaChrom system (Merck, Poole, Dorset, UK) equipped with an APEX Carbohydrate column (Jones Chromatography, Mid Glamorgan, UK) and a Merck-Hitachi LaChrom RI detector. 70% (v/v) acetonitrile was used as an eluent at 25°C and a flow rate of 0.8 ml/min. The galactooligosaccharide mixture comprised of 25% Gal-Gal, 35% Gal-Gal-Glc, 24 % Gal-Gal-Gal-Glc and 16% Gal-Gal-Gal-Gal-Glc.

EXAMPLE 2

Bifidobacterium bifidum NCIMB 41171 fixed cells were prepared according to Example 1 and added to 500ml of skimmed milk in a stirred tank, added (300 units of β-galactosidase activity). Lactose conversion was allowed to proceed at 40°C. After 8h the galactooligosaccharides concentration was 22% (w/v) and the mixture comprised 28% Gal-Gal, 32% Gal-Gal-Glc, 21% Gal-Gal-Gal-Glc and 19% Gal-Gal-Gal-Gal-Glc.

EXAMPLE 3

Bacterial strains and culture conditions

Eschericia coli O157 VT⁻, *E. coli* 11775 and *Salmonella typhimurium* were grown in Luria-Bertani broth (BBL) at 37°C for 18-20h before use. Before the adhesion assay, the bacteria were labelled with fluorescein 5-isothiocyanate (FITC, Sigma) by gently stirring in phosphate buffered saline (PBS, pH 7.9) containing 0.5% FITC for 3 hours at 4°C. The bacteria were pelleted by centrifugation at 3000xg for 10 minutes and then washed three times with PBS. FITC-conjugated bacteria were suspended in PBS at a cell density of $1x10^8$.

Intestinal cell culture

The human intestine adenocarcinoma cell line, Caco-2, were cultured in Dulbecco's modified Eagle's minimal essential medium, containing 25mM glucose, 20% (v/v) heat-inactivated foetal calf serum, and 1% (w/v) non-essential amino acids. Cells were grown at 37°C, in 5% v/v CO_2 in air. For the adhesion assay, monolayers of Caco-2 cells were prepared in 24-well tissue culture dishes by inoculating $1x10^5$ viable cells per well in 1.0 ml of culture medium. The medium was replaced every 2 days.

Carbohydrates tested

The carbohydrates tested were: galactose, glucose, lactose, and the synthesised galactooligosaccharide (GOS) mixture produced by the action of *B. bifidum* (NCIMB 41171) enzymes. A concentrated solution of the respective carbohydrate was added to a suspension of the bacterium in PBS (pH 7.9) to give a 25mM carbohydrate solution. The assay was then performed in the presence of the respective carbohydrate.

Adhesion assay

Caco-2 cell monolayers were washed once with 1.0 ml of sterile PBS (pH 7.8) before the adhesion assay. FITC-conjugated bacteria at concentration of $1x10^8$ were added to each well in 1.0 ml (total volume) of PBS (pH 7.9) and incubated at 37°C, in 5% CO_2 in

air, with gentle rocking. After incubation for 30 minutes the monolayers were washed three times with sterile PBS to remove free bacterial cells. The concentration of adhered bacterial cells was estimated in a flow cytometer equipped with an air-cooled 488-nm argon-ion laser at 15mW.

The adhesion rate of the bacteria on Caco-2 cells was calculated by the following equation:

Adhesion rate (%) = (Fluorescence of cells assayed in the presence of tested carbohydrate) / (Fluorescence of cells assayed in the absence of carbohydrate)

Results

Adhesion rate (%) of bacteria on Caco-2 cells in the presence of carbohydrates. The control was performed in the absence of carbohydrate

Bacterium	**Control**	**Glucose**	**Galactose**	**Lactose**	**GOS**
Eschericia coli O157 VT$^-$	1.00	0.75	0.74	0.72	0.55
E. coli 11775	1.00	0.84	0.82	0.79	0.66
Salmonella typhimurium	1.00	0.86	0.85	0.84	0.74

Conclusion

Using the galactooligosaccharide mixture produced by *B. bifidum* (NCIMB 41171) in Example 2 the adhesion rate of bacteria on Caco-2 cells was lowered significantly.

EXAMPLE 4

***In vitro* gut model**

The conditions in the colon were replicated in a three stage continuous fermenter (Macfarlane *et al.*, 1998, Microbial Ecology, 35, 180-187) inoculated with 10% (w/v) faecal homogenate from healthy human volunteers in a growth medium without and with 1% (w/v) the GOS mixture (Table 2). The model consisted of three vessels, V1, V2 and V3,

with respective operating volumes of 270, 300 and 300 ml. Temperature was set at 37°C and together with pH was controlled automatically. Culture pH in the three vessels was maintained at 5.5, 6.2 and 6.8, respectively. Each fermenter was magnetically stirred and kept under anaerobic conditions by continuously sparging with O_2-free N_2 (15ml/min). The growth medium contained the following ingredients: starch 8g/l, mucin 4g/l, casein 3g/l, peptone water 5g/l, tryptone water 5g/l, bile N°3 0.4g/l, yeast, 4.5 g/l, $FeSO_4$ 0.005g/l, NaCl 4.5g/l, KCl 4.5g/l, KH_2PO_4 0.5g/l, $MgSO_4.7H_2O$ 1.25g/l, $CaCl_2.6H_2O$ 0.15g/l, $NaHCO_3$ 1.5g/l, Tween 80 1ml, Hemin 0.05g/l, Cysteine.HCl 0.8g/l. The medium was fed to V1 by a peristaltic pump and V1 sequentially supplied V2 and V3 through a series of tubes. The system was operated at a retention time of about 36 hours. The gut model was left overnight to equilibrate before the medium pump was switched on and was run for at least 10 days before medium containing testing substrate was introduced and it was then left for further 10 days. Samples were taken at the beginning and the end of each cycle. The sample volume removed was 5 ml and this amount was used for bacterial group enumeration.

Fluorescence *in situ* hybridisation (FISH)

Differences in bacterial populations were assessed through use of FISH with oligonucleotide probes designed to target diagnostic regions of 16S rRNA. These were commercially synthesised and labelled with the fluorescent dye Cy3 (provided by Eurogentec UK Ltd). The molecular probes utilised were presented in Table 1. For total bacterial counts the nucleic acid stain 4,6-diamidino-2-phenylindole (DAPI) was used. Samples obtained from fermentation vessels were diluted in 4% (w/v) paraformaldehyde and fixed overnight at 4°C. The cells were then centrifuged at 1500 x g for 5 minutes, washed twice with phosphate-buffered saline (PBS; 0.1M, pH 7.0), resuspended in a mixture of PBS / 99% ethanol (1:1 w/v) and stored at –20°C for at least 1 hour. The cell suspension was then added to the hybridisation mixture and left overnight to hybridise at the appropriate temperature for each probe. Hybridised mixture was vacuum filtered using a 0.2µm Isopore membrane filter (Millipore Corporation, Herts, UK). The filter was removed, placed onto a glass slide with SlowFade (Molecular Probes, Eugan, OR, USA) and examined under a fluorescent microscope (Nicon Eclipse, E400). The DAPI stained

cells were examined under UV light and hybridised cells viewed using a DM510 filter. For each slide at least 15 different fields of view were counted.

Table 1. Oligonucleotide probes used for the characterisation of gut microflora using FISH

Probe	Sequence	Target genus	Temperature	Reference
Bac 303	5'-CCAATGTGGGGGACCTT-3'	*Bacteroides* spp.	45°C	Langendijk *et al.* (1995)
Bif 164	5'-CATCCGGCATTACCACCC-3'	*Bifidobacterium* spp.	50°C	Manz *et al.* (1996)
Chis 150	5'-AAAGGAAGAUUAAUACCGCAUA-3'	*Clostridium histolyticum* group	50°C	Franks *et al.* (1998)
Lab 158	5'-GGTATTAGCA(T/C)CTGTTTCCA-3'	*Lactobacillus/Enterococcus* spp.	45°C	Harmsen *et al.* (1999)

RESULTS

Table 2. Bacterial populations as determined by FISH in an *in vitro* gut model when commercial GOS (Vivinal (RTM)) was used as a substrate at 7g per day.

	V1			V2			V3		
Time (days)	1	10.5	21	1	10.5	21	1	10.5	21
Total bacteria (log no.)	9.5	9.5	9.6	9.5	9.4	9.5	9.5	9.4	9.6
Bifidobacterium spp.	8.0	7.9	8.3	8.0	8.0	8.3	8.0	8.0	8.2
Lactobacillus spp	7.2	7.2	7.1	7.0	7.0	7.1	7.0	7.0	6.9
Bacteroides spp	8.1	8.1	7.5	8.0	8.2	7.5	8.0	8.1	7.9
Clostridium histolyticum group	6.8	6.9	7.1	6.9	6.8	7.0	6.9	6.8	7.0

Table 3. Bacterial populations as determined by FISH in an *in vitro* gut model when the synthesized GOS of the present invention was used as a substrate at 7g per day.

	V1			V2			V3		
Time (days)	1	10.5	21	1	10.5	21	1	10.5	21
Total bacteria (log no.)	9.4	9.7	9.6	9.4	9.5	9.6	9.6	9.5	9.5
Bifidobacterium spp.	8.1	8.0	8.9	8.0	8.0	8.7	8.2	8.2	8.4
Lactobacillus spp	7.4	7.6	7.6	7.3	7.3	7.5	7.4	7.3	7.3
Bacteroides spp	8.0	8.2	8.2	8.0	8.1	8.1	7.8	7.8	7.8
Clostridium histolyticum group	6.9	7.0	6.8	6.8	6.8	6.7	7.0	7.0	6.9

CONCLUSION

From Table 3, it can be seen that the GOS mixture of the present invention shows better prebiotic properties, (i.e. a higher increase in bifidobacteria, as well as a decrease in bacteroides than the commercial GOS equivalent (see Table 2). The prebiotic effect was stronger in vessel 1 (V1) and 2 (V2), which is explained by the fact that our GOS consists of low molecular weight oligosaccharides.

EXAMPLE 5

Utilisation of infant rhesus macaques to model the infant human gut and to test the effect of various milk supplements on faecal bacteriology.

Materials and Methods

25 infant Rhesus Macaques (*Maccaca mulatta*) were reared at the California Regional Primate Research Centre at the University of California, Davis, USA and divided into 5 groups of 5 animals each. Each animal is housed individually in polycarbonate isolettes with a surrogate mother (O to 1 months) before being paired in stainless steel cages (1 to 5 months). The groups are fed experimental diets of breast milk, infant formula containing either GOS (the prebiotic) or glycomacropeptide (GMP, dry-blended), whey-predominant formula, or skim milk powder. All products and formulas contain about 27% (w/w) fat, 9.6% protein and 56% carbohydrates. Rectal swabs are collected once a week for a period of 5 months. At 4.5 months, an infectious dose of 10^8 cfu/ml of the Enteropathogenic (EPEC) *Escherichia coli* O127 strain 2348/69 is given. All animals are swabbed immediately before *E.coli* dosing (day 0) and at days 1, 4 and 7 after dose. After swabbing samples are immediately frozen and eventually transported (average time 6 months) for processing and analysis of the bacteria by fluorescence in situ hybridisation (FISH).

For the sample preparation, 3ml of phosphate buffered saline, PBS, (pH7.4)

containing 0.00001% (w/v) cetyl trimethyl ammonium bromide (CTAB) is added to each swab within its sample tube and shaken vigorously for 10 minutes on an orbital shaker to bring the sample into solution. The swab is discarded and the entire sample transferred to a 50ml centrifuge tube (Nalgene, Rochester, NY) containing 9ml of filter sterilised 4% (w/v) paraformaldehyde in PBS (pH 7.2) and fixed for at least 4 hours at 4°C. The fixed sample is centrifuged for 15 minutes at 13,000xG and the supernatant discarded. The pellet is re-suspended in 1ml of filtered PBS (pH 7.4) and transferred into a 1.5ml Eppendorf tube for repelleting (13,000xG, 10 min.). After washing the pellet a second time, the supernatant is removed and the pellet re-suspended thoroughly in 150µl of filtered PBS and 150µl of 96% (v/v) ethanol. The sample is mixed well and stored at -20°C for at least 1h before further processing.

Oligonucleotide probes for *Bifidobacterium* (5'-CAT CCG GCA TTA CCA CCC-3'), *Lactobacillus / Enterococcus* (5'-GGT ATT AGC ATC TGT TTC CA-3'), and *Escherichia coli* (9) (5'-CAC CGT AGT GCC TCG TCA TCA-3') are synthesised and monolabelled at the 5' end with Cy3 (Ex 552nm, Em 568nm) by either Eurogentec (Abingdon, UK) or MWG-Biotech (Milton Keynes, UK). Hybridisation is carried out overnight at 50°C for *Bifidobacterium*, 45°C for *Lactobacillus* and 37°C for *E.coli*.

For hybridisation, 200µl of filtered 2x hybridisation buffer (40mM Tris-HCl pH 7.2, 1.8M NaCl) containing 20ml/l 10% (w/v) SDS and 64µl of HPLC grade water are added to 16µl of fixed cells and warmed to the appropriate hybridisation temperature. For the *E.coli* probe, 264µl of 2x hybridisation buffer containing 35%(v/v) formamide added to 16ml of fixed cells are used for the hybridisation mixture. 90µl of the pre-warmed hybridisation solution is added to 10µl of appropriate probe (final concentration 50ng/µl) and the entire solution is returned to the hybridisation oven and left overnight.

For washing the sample and obtaining total bacterial counts, using 4',6-diamidino-2-phenylindole (DAPI, Ex 344, Em 450), 5ml of 1x hybridisation buffer (20mM Tris-HCl pH 7.2, 0.9 M NaCl) and 20µl DAPI (500ng/ml) are added to 5-100µl of hybridised sample

and placed in the hybridisation oven for 30 minutes. The actual amounts varied according to cell densities obtained in each sample. For total counts, 5-12µl of sample are used. The washed sample is vacuum filtered onto a 0.2µm pore-size polycarbonate filter (Millipore, Watford, UK) and placed onto a microscope slide. To avoid fading of the probes, one drop of SlowFade™-Light Antifade Kit component A (Molecular Probes Europe BV, Leiden, The Netherlands) is added to the filter and covered with a cover slip. To further minimise fading, the slides are stored in the dark at 4°C until further use.

For counting the bacteria, slides were placed onto a fluorescence microscope (Leitz, Wetzlar, Germany). UV light was used for counting DAPI stained bacteria and Cy3 stained cells were assessed at 550nm. 15 random fields (92µmx92µm) with a good distribution of cells were counted for each probe and sample.

Results

Breast-fed animals and those receiving GOS (the prebiotic) supplemented formula have populations of lactobacilli, bifidobacteria, and *E.coli* that fluctuated throughout the course of the diet. However, all populations stay within 1 log value of the initial counts. Animals fed either GMP or control formula show no significant changes in *Lactobacillus* counts but exhibit fluctuations in *E.coli* and bifidobacteria.

Animals on formula supplemented with GOS (the prebiotic) and breast-fed animals have decreasing *E.coli* and *Lactobacillus* populations after EPEC dosing. There is no observable change in the other groups. This decrease is unrelated to the incidence of diarrhoea and animals receiving the control formulae to get acute diarrhoea. Animals receiving GMP supplemented formula and breast-fed infants have intermittent diarrhoea whereas animals taking GOS supplement have no diarrhoea. After EPEC inoculation, levels of bifidobacteria increased modestly in animals taking the GOS and GMP supplement. In comparison, there is only a minimal increase in breast-fed and control formula-fed animals.

Conclusion

Diarrhoea causing gastrointestinal diseases are one of the major causes of infant morbidity and mortality in the developing world with EPEC amongst the more common of the associated pathogens. Diet and the infant's immunocompetence are reasons why pathogens are able to survive and colonise the gastrointestinal tract. Breast milk contains many components that aid in colonisation resistance by promoting a beneficial, protective microflora as well as providing antibacterial properties.

In this study, we show that breast-fed infant macaques and those fed GOS (the prebiotic) supplemented whey predominant formula have no incidence of diarrhoea when infected with EPEC. *Bifidobacterium* populations modestly increase, indicating that GOS (the prebiotic) has a bifidogenic effect. Similar effects have been observed in breast milk mostly due to lactoferrin, which proliferates *Bifidobacterium infantis, B.breve and B.bifidum in vitro*.

EXAMPLE 6

Methylation analysis

Galactooligosaccharide synthesis products prepared according to Example 1 were purified by gel filtration on a column of Biogel P2 (Pharmacia) eluted at 3 ml min^{-1} with water.

Linkage positions for the respective galacto-oligosaccharides preparations were determined by methylation analysis. The freeze-dried samples (5-6mg) were dispersed in dry dimethyl-sulfoxide (DMSO) at 20°C for 16 h after flushing with argon. They were methylated by sequential addition of powdered sodium hydroxide (0.5g) and iodomethane (4ml) (Ciucanu and Kerek, 1984; MacCormick et al, 1993). After elution-extraction on a C18-bonded cartridge (Sep-Pak, Waters, Watford, UK), the methylated carbohydrates were

dried, extracted into $CHCl_3/CH_3OH$ (1:1, v:v), and evaporated to dryness. The samples were hydrolysed using trifluoroacetic acid (Blakeney et al, 1983), and converted to partially methylated alditol acetates (PMAAs) by $NaBD_4$ reduction and acetylation with acetic anhydride and N-methylimidazole (Alberscheim et al, 1967).

The PMAAs were analysed by GC on a cross-bonded 50% cyanopropyl methyl-50% phenyl methyl polysiloxane column (Thames Chromatrography, Maidenhead, UK) using a flame ionisation detector and a temperature program: 55°C (2 min), + 45°C min^{-1} (1.9 min), 140°C (2min), + 2°C-min^{-1} (35 min), 210°C (40 min). The PMAAs were identified by measuring their retention times relative to *myo*-inositol hexaacetate, and comparing the relative retention times with those of external standards. A mixture of standards for each sugar was prepared by deliberate methylation of methyl glycosides (Doares et al, 1991). Peak areas were represented as relative molar quantities using effective carbon response factors (Sweet et al, 1975).

Identities of PMAAs were confirmed by their electron-ionisation mass spectra (Carpita and Shia, 1989). GC-MS analysis was performed on an identical GC in series with a Fisons Analytical Trio 1S mass spectrometer, using a source temperature of 200°C and an ionization potential of 70eV.

In order to determine the anomeric configuration of the synthesis product, the oligosaccharides were treated with α-Galactosidase or β-Galactosidase (Melibiase; Sigma) at the optimum conditions suggested for 30 min. The reaction products were analysed by HPLC.

Results

From the above analysis the oligosaccharide structure was estimated to be for the tetrasaccharide fraction Gal (β 1-6)- Gal (β 1-6)- Gal (β 1-4)- Glc, the trisaccharide fraction Gal (β 1-6)- Gal (β 1-4)- Glc; Gal (β 1-3)- Gal (β 1-4)- Glc and the disaccharide fraction Gal (β 1-4)- Glc (lactose substrate); Gal (β 1-3)- Glc; Gal (β 1-3)- Gal; Gal (β 1-6)- Gal;

Gal (α 1-6)- Gal (galibiose)

Gal: galactose, Glc: glucose

5 References

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various GLC response-factor theories for partially methylated and partially ethylated alditol acetates. Carbohydr Res **40**: 217-225

EXAMPLE 7

Materials and Methods

The HT29 cell line was obtained from European Collection of Cell Cultures for Applied Microbiology and Research. Cell stocks were cultured at 37°C in humidified 5%CO_2 in a standard medium containing high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) foetal bovine serum (FBS), 100 mM penicilin, 0.1M streptomycin, non essential amino acids (NEAA x100) and 200mM a-glutamine. Cells were re-fed every 48h and passaged before confluence was reached.

Oligosaccharide sensitivity assay

Serum standard medium (1% v/v) supplemented with different concentrations of oligosaccharides (0.01, 0.1, 1, 10, 100 mM) were used for oligosaccharide sensitivity assay according to Olano-Martin et al., 2003). Cells were re-fed experimental medium (containing the oligosaccharide of interest) daily, and measurement of adherent cells was performed by removal of experimental media and washing off the cells with Ca^{++} free phosphate buffered saline (pH 7, 9.6 gL^{-1}). Adherent cells were then trypsinised and neutralised with an equal volume of serum standard medium. The cell suspension was diluted in Isoton II and cells were counted in a Coulter Counter. Percentage of cell survival was calculated as follow (Figure 1)

% survival =(mean absorbance of treated cells/mean absorbance of control) x 100

Adhesion assay

HT29 cell were grown in 12-well tissue culture plates to >90% confluence using

standard medium. For the last cell feeding prior to performing the assay, antibiotic-free medium was used.

Pathogens were grown anaerobically in antibiotic free cell culture medium for at least three subcultures. On the day of the assay, fresh pre-reduced tissue culture medium was inoculated with 10% of an overnight pathogen culture and incubated for 4h prior to the assay.

Stock solution of the test oligosaccharides was prepared at a concentration of 5M in phosphate buffer saline and filter sterilised.

A 1/1000 dilution of the 4h pathogen culture, was prepared in PBS and enumerated by plate counting. The medium was apirated off from the tissue culture plate and the cells were washed once in PBS (1ml).

For each test oligosaccharide, 0.5 ml oligosaccharide (5M) solution was added to three wells. Phospate buffer saline (PBS) without any oligosaccharide was included as control. 0.5 ml of culture suspension was added to all wells, the plate was rock mixed and incubated aerobically at 37°C for 2h.

The culture was aspirated off, and all wells were washed three times in sterile PBS (1ml per well). After the final washing, PBS was aspirated off and 70µl trypsin/EDTA solution was added to each well, mixed and let stand for 5 minutes at 37°C.

1ml PBS was added per well and pipette mixed to ensure that all the cells were removed from the bottom of the well and that clumps were broken up.

1ml of the cell suspension was pipetted into a universal bottle of MRD and further diluted as appropriate. Dilutions were plated out on plate count agar (PCA) and incubated at 37°C for 24h.

After incubation colonies were enumerated and inhibition of adhesion was calculated as the ratio of bacteria (c.f.u ml^{-1}) present in the sample to control (PBS) (Figure 2).

5 Conclusion

The results shown in Figure 2 indicate a strong inhibition of adhesion of E. coli EPEC and S. typhimurium in the presence of the disaccharide fraction, which inhibition is also present in the GOS as a mixture. There is a lower anti-adhesion effect in the presence of the higher than trisaccharide fraction of the mixture against S. typhimurium.

The oligosaccharide sensitivity assay is performed to assure that are oligosaccharide mixture is not toxic to the HT29 cells (Figure 1).

References

Olano-Martin E., Williams M.R., Gibson G.R., Rastall R.A. 2003. Pectins and pectic-oligosaccharides inhibit Escherichia coli O157 : H7 Shiga toxin as directed towards the human colonic cell line HT29. FEMS Microbiol Letters 218 (1): 101-105

Figure 1. Cell survivability as affected by the addition of different oligosaccharide concentrations (0.01-100mM) after 24 and 48h of incubation.

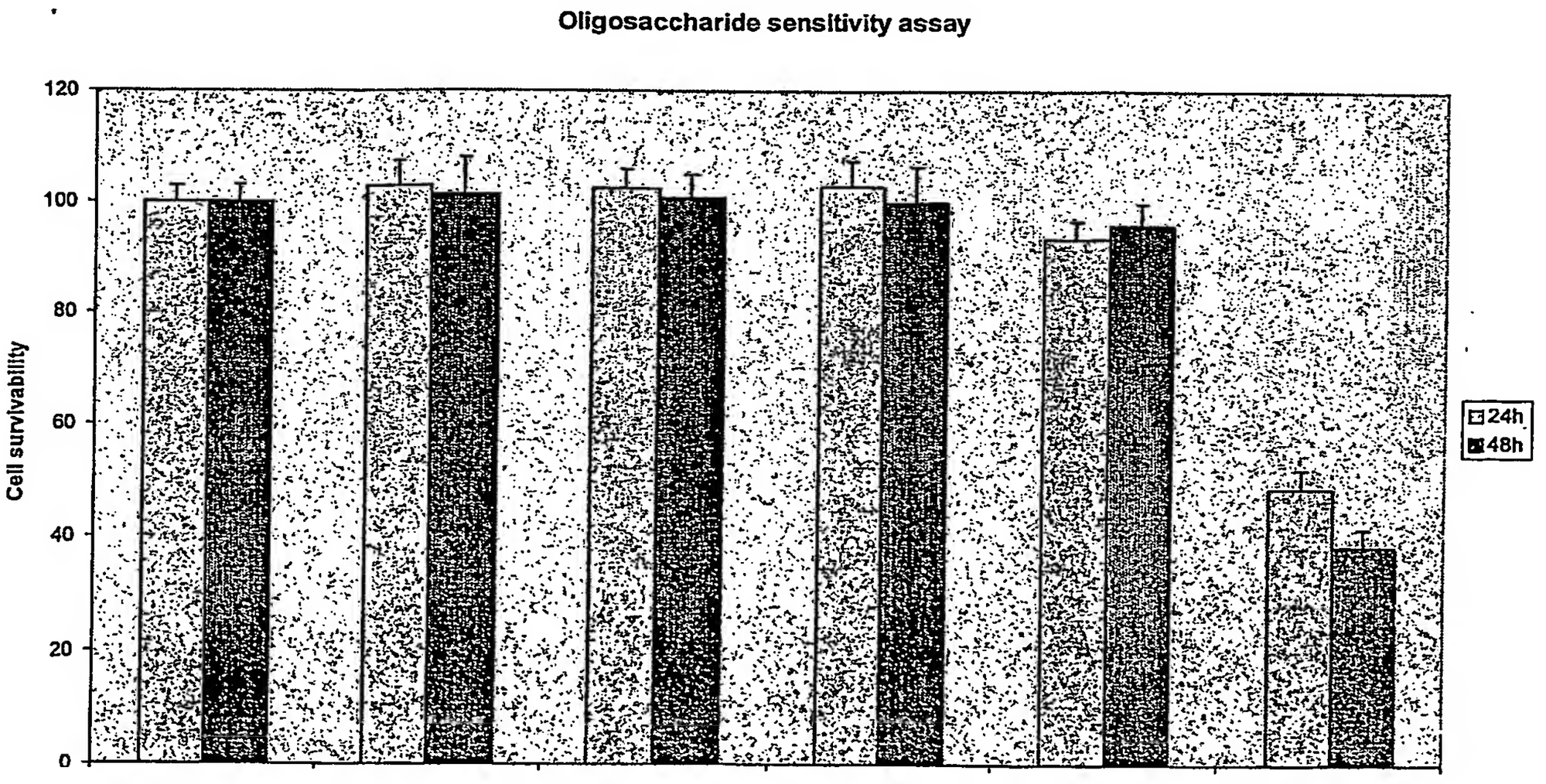

Figure 2. Effect of Oligosaccharide mixture (ALL) and of the different fractions of the mixture on the adhesion of E. coli EPEC, E.coli VTEC and Salmonella typhimurium to HT29 cells.

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